PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : C07K 16/00

A2

(11) International Publication Number: **WO 99/36440**

(43) International Publication Date: 22 July 1999 (22.07.99)

(21) International Application Number: PCT/GB99/00152

(22) International Filing Date: 18 January 1999 (18.01.99)

(30) Priority Data:
9800927.7 16 January 1998 (16.01.98) GB

(71) Applicant *(for all designated States except US)*: UNIVERSITY OF BRISTOL [GB/GB]; Senate House, Tyndall Avenue, Bristol BS8 1TH (GB).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: HAWKINS, Robert, Edward [GB/GB]; Christie CRC Research Centre, Wilmslow Road, Manchester M20 4BX (GB). WHITTINGTON, Hayley, Ann [GB/GB]; University Dept. of Oncology, Bristol Oncology Centre, Horfield Road, Bristol BS2 8ED (GB).

(74) Agents: DEAN, John, Paul et al.; Withers & Rogers, Goldings House, 2 Hays Lane, London SE1 2HW (GB).

(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, HR, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published
Without international search report and to be republished upon receipt of that report.

(54) Title: EXPRESSION OF FUSION PROTEINS

(57) Abstract

This invention provides a method of producing a fusion protein comprising an antibody portion and a further biologically active portion, the method comprising forming a virus–based construct including a DNA sequence encoding the fusion protein and infecting cells or an organism with the thus constructed virus–based construct whereby the fusion protein is expressed in *in vitro* in the cells, or *in vivo* in the organism.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	**ES**	Spain	**LS**	Lesotho	**SI**	Slovenia
AM	Armenia	**FI**	Finland	**LT**	Lithuania	**SK**	Slovakia
AT	Austria	**FR**	France	**LU**	Luxembourg	**SN**	Senegal
AU	Australia	**GA**	Gabon	**LV**	Latvia	**SZ**	Swaziland
AZ	Azerbaijan	**GB**	United Kingdom	**MC**	Monaco	**TD**	Chad
BA	Bosnia and Herzegovina	**GE**	Georgia	**MD**	Republic of Moldova	**TG**	Togo
BB	Barbados	**GH**	Ghana	**MG**	Madagascar	**TJ**	Tajikistan
BE	Belgium	**GN**	Guinea	**MK**	The former Yugoslav Republic of Macedonia	**TM**	Turkmenistan
BF	Burkina Faso	**GR**	Greece			**TR**	Turkey
BG	Bulgaria	**HU**	Hungary	**ML**	Mali	**TT**	Trinidad and Tobago
BJ	Benin	**IE**	Ireland	**MN**	Mongolia	**UA**	Ukraine
BR	Brazil	**IL**	Israel	**MR**	Mauritania	**UG**	Uganda
BY	Belarus	**IS**	Iceland	**MW**	Malawi	**US**	United States of America
CA	Canada	**IT**	Italy	**MX**	Mexico	**UZ**	Uzbekistan
CF	Central African Republic	**JP**	Japan	**NE**	Niger	**VN**	Viet Nam
CG	Congo	**KE**	Kenya	**NL**	Netherlands	**YU**	Yugoslavia
CH	Switzerland	**KG**	Kyrgyzstan	**NO**	Norway	**ZW**	Zimbabwe
CI	Côte d'Ivoire	**KP**	Democratic People's Republic of Korea	**NZ**	New Zealand		
CM	Cameroon			**PL**	Poland		
CN	China	**KR**	Republic of Korea	**PT**	Portugal		
CU	Cuba	**KZ**	Kazakstan	**RO**	Romania		
CZ	Czech Republic	**LC**	Saint Lucia	**RU**	Russian Federation		
DE	Germany	**LI**	Liechtenstein	**SD**	Sudan		
DK	Denmark	**LK**	Sri Lanka	**SE**	Sweden		
EE	Estonia	**LR**	Liberia	**SG**	Singapore		

Expression of Fusion Proteins

This invention relates to the delivery of fusion proteins comprising antibodies or recombinant fragments thereof to organs, tissues, cells and the like. More particularly, the invention relates to use of virus based, constructs to deliver fusion proteins comprising antibody fragments thereof to target tumour cells where the fusion proteins are exposed.

It is known in the art that antibodies have enormous potential for the therapy of malignant and other diseases. For the therapy of malignant disease, the use of small fragments of antibodies, and fusion proteins, derived from them has potential advantages (Chester K.A. and Hawkins R.E. Advanced Drug Delivery Reviews. 1996; **22**: 303-314 and Chester K.A. and Hawkins R.E. TIBTECH 1995;**13**: 294-300). A particular form of antibody fragments which has been extensively studied in recent years is the single chain Fv ("scFv") (Huston J.S. *et al*, Proc. Natl. Acad.Sci. USA 1988; **85**:5879-5883). These fragments have great potential for therapy as they are small and penetrate tumours and solid tissues relatively well. They can be isolated directly using phage technology (Hawkins R.E. *et al* J. Mol. Biol. 1992; **226**:889-896) and can be subsequently engineered to enhance their affinity (Chester K.A., *et al* Lancet 1994; **343**: 455-456). Indeed such fragments made from phage libraries (Verhaar M.J., *et al* Int J Cancer 1995; **61**: 497-501) appear to be superior to those derived from pre-existing monoclonal antibodies in tumour targeting models (Begent R.H.J., *et al* Nature Medicine 1996; **2**: 979-984). Recently their activity in patient imaging studies has been established and this work also confirms the short half-life of such antibody fragments in the human circulation.(Savage P., *et al* Br. J. Cancer 1993; **67**: 304-310.) Fusion proteins with such scFv fragments are potentially attractive for therapeutic use but the short half life of the antibody fragments means that continuous infusion for a period of time may be required to obtain sufficient target tissue concentrations and that large quantities will need to be administered (see Hawkins *et al supra*).

Difficulties in the production and purification of bulk quantities of fusion protein remain. Hitherto, the fusion proteins have been made *in vitro* using hybridoma technology. It is

therefore an object of the present invention to provide an attractive and alternative method for the production of antibody fragments to overcome disadvantages of known techniques.

According to a first aspect of the invention, there is provided a method of producing a fusion protein comprising an antibody portion and a further biologically active portion, the method comprising forming a virus-based construct including a DNA sequence encoding the fusion protein and infecting cells or an organism with the thus constructed virus-based construct, whereby the fusion protein is expressed in *in vivo* in the cells, or *in vitro* in the organism.

Preferably the antibody portion or fragment, or construct, is fused to GM-CSF so that it is able to function as a cytokine in order to induce a systemic immune response. The use of GM-CSF is particularly attractive since, as well as having a direct effect on targeted cell killing, it is a particularly effective cytokine for the induction of systemic immune responses to tumour cells.

There are a number of different fusion proteins which could be used (see Chester K.A and Hawkins R.E. *supra*) but antibody-cytokine fusion molecules are preferably used because of their potential to recruit specific effector cells. Such fusion proteins, produced using hybridoma technology, have previously been described with regard to use with interleukin-2 (Hornick J.L., *et* al. Blood 1997; **89**: 4437-4447) and with GM-CSF (Dranoff G., *et al. Proc. Natl. Acad. Sci.* U S A 1993; **90**: 3539-3543.and Lee C.T., *et al.* Hum. Gene Ther. 1997; **8**: 187-193). This is achieved by altering the local immunological environment to increase the immunogenicity of the tumour cells (Lee C.T., *et al.* Hum Gene Ther 1997; **8**: 187-193). Practically, however, it is preferable to treat tumours directly *in vivo*. Systemic toxicity means that it is necessary to devise methods that increase the concentration of cytokine in the immediate vicinity of the tumour. One approach has been to use gene transfer to introduce cytokine genes into tumour cells *in vivo*. Recombinant adenovirus has been used to introduce the GM-CSF gene into a murine lung tumour model system and to suppress growth of the tumour cells (Stevenson F. K., *et al.* Immunol Rev 1995; **145**: 211-228). However, tumours are often not readily accessible for the direct introduction of adenoviruses and in the adjuvant setting (where immunotherapy

is most likely to be effective) all known tumour has been removed so it is generally preferable to target the cytokine itself if appropriate methods can be devised.

It will be appreciated that the recombinant adenoviruses encoding scFv fusion proteins as a model for the production of antibodies and their fragments *in* vivo overcome practical problems associated with the production and clinical use of antibodies and scFvs for immunotherapy of cancer. The recombinant adenoviruses,Y.B1.8.mGM-CSF and Y.MFE-23.mGM-CSF,(Ψ.B1.8.mGM-CSF and Ψ.MFE-23.mGM-CSF) have been generated by a method that utilises Cre recombinase (Hardy S *et al* J Virol 1997; 71: 1842-1849) rather than relying on the more inefficient process of overlap recombination (Bett AJ *et al* Proc Natl Acad Sci U S A 1994; 91: 8802-8806). They contain a gene fusion encoding murine GM-CSF fused to the scFvs B1.8 or MFE-23, which bind to the antigens NIP or CEA, respectively. These adenoviruses are able to infect a variety of human and murine cell lines *in vitro* and promote expression and secretion of the scFv.mGM-CSF proteins. In the case of MFE-23.mGM-CSF, the presence of CEA on the cell surface in a number of these lines does not appear to have a major effect on secretion of the fusion protein. This suggests that secretion of the fusion protein is not prevented by aggregation with or binding to the CEA intracellularly. Importantly, both parts of the scFv.mGM-CSF fusion proteins are bioactive. The scFvs are able to bind specifically to their respective antigens *in vitro* and the mGM-CSF domain promotes *in vitro* proliferation of the murine myeloid cell line FDC-P1, which is dependent on either GM-CSF or IL-3 for growth. Quantitive assessment of the mGM-CSF activity suggests full biological activity is retained and importantly no significant proteolysis can be detected. The Y.MFE-23.mGM-CSF virus when injected intravenously into a mouse infects murine cells efficiently enough to produce substantial amounts of the fusion protein. The MFE-23.mGM-CSF protein can be detected in mouse serum by ELISA for at least 13 days post infection, which is in accordance with previous data for the expression of proteins from adenoviral vectors *in vivo* (Kozarsky KF *et al* J Biol Chem 1994; **269**: 13695-13702).

One of the potential advantages of using an antibody-GM-CSF fusion protein for therapy in comparison to systemic GM-CSF administered alone or in conjunction with a monoclonal antibody, is that it should be possible to achieve high concentrations of GM-CSF in the

immediate vicinity of the targeted cells but maintain low levels of systemic GM-CSF hence reducing toxicity. However, these proteins have a short half life in the body. ScFvs and Fab' fragments (which have a similar molecular weight to scFv.mGM-CSF protein) have a half-life in plasma of 2.8 and 7.5 hours, respectively (Colcher D *et al* J Natl Cancer Inst 1990; **82**: 1191-1197). An antibody-GM-CSF fusion protein has a half-life of approximately 30 hours (Dranoff G., *et* al. *Proc. Natl. Acad. Sci* USA 1993; **90**: 3539-3543). Hence, it is likely that repeated administration would be required to maintain effective therapeutic levels. Using a recombinant adenovirus to direct expression of these proteins *in vivo* should allow low levels of expression to be maintained over a longer period of time. Levels of MFE-23.mGM-CSF exceeding 1000 ng/ml serum have been produced in mice. These levels induce fatal toxicity, causing leukocytosis, hepatosplenomegaly and pulmonary haemorrhage (Dranoff G *et al* Proc Natl Acad Sci U S A 1993; **90**: 3539-3543) and in this experiment resulted in sacrifice of such mice due to toxicity. Lower doses of recombinant virus produce lower potentially therapeutic but non-toxic levels (< 25 ng/ml serum) of the protein.

There are a number of strategies which could allow longer expression from each administration and effective repeated use of recombinant adenoviruses if that is required.

One approach to prolong the expression of the fusion protein *in vivo* would be to use a retrovirus particularly a lentivirus. Lentiviruses have been used to achieve prolonged and substantial expression of proteins compared to adenovirus based systems (Kafric *et al* Nature Genetics **17** Nov. 97).

Certainly, the requirement for repeated administration of adenovirus in therapy protocols has prompted studies on ways of reducing the host immune response to the adenovirus and hence extending the period of transgene expression. Immunomodulatory agents that have an antagonistic effect on the immune response have been studied (Lei D *et al* Hum. Gene Ther. 1996; **7**: 2273-2279, Kay MA *et al* Nat. Genet. 1995; **11**: 191-197). Second generation adenoviral vectors containing further deletions of adenoviral genes encoding structural proteins have also been constructed. This markedly increases the period of transgene expression and highlights a role for adenoviral protein expression in eliciting a host immune response (Engelhardt JF *et al* Proc Natl Acad Sci U S A 1994; **91**:

6196-6200). The most extreme of these adenoviral vectors have all adenoviral coding sequences deleted (Kochanek S *et al* Proc Natl Acad Sci U S A 1996; **93**: 5731-5736, Fisher KJ *et al* Virology 1996; **217**: 11-22, Hardy S *et al* J Virol 1997; **71**: 1842-1849). They require a helper virus for the production of adenoviral particles. The absence of adenoviral protein expression in infected cells *in vivo* increases the persistence of the adenovirus. Studies in animals tolerised to the transgene product have shown that expression from such vectors can last for >80 days post infection. In non-tolerised animals, expression was lost by 42 days (Chen HH *et al* Proc Natl Acad Sci U S A 1997; **94**: 1645-1650). The use of these type of adenoviral vectors may allow us to achieve expression of scFv.GM-CSF proteins for a longer period *in vivo*. The level of protein produced may also be controlled by the amount of virus administered relative to body weight and also by the incorporation of gene regulatory sequences (Hu SX *et al* Cancer Res 1997; **57**: 3339-3343). The recently described positive feedback tetracycline control system may be particularly effective when used in constructs in accordance with the invention (Agha-Mohammedi S & Hawkins RE Gene Therapy 1997 in press).

These experiments show that is possible to express a scFv with therapeutic potential at levels which are biologically active and sustained for around 13 days. This approach has a number of theoretical and practical attractions and may be used in animals for anti-tumour activity. In the future it may be possible to extend this system as indicated above to allow repeated administration and also to target the expression to organs of interest. For example, physical means could be used to direct expression to the liver (a common site of colorectal cancer metastasis) or the peritoneum (a common site of ovarian cancer metastasis) to obtain higher levels of scFv fusion proteins where they are most needed. It may also be possible to target expression of the scFv fusion to specific tissues either using tissue specific promoters (Rothmann T *et al* Gene Ther 1996; 3: 919-926) in the constructs of the invention or by targeting gene delivery (Watkins SJ *et al* Gene Therapy 1997; 4: 1004-1012). An approach to targeting certain tissues would be to remove relevant cells from an organism infect them with a virus based construct *in vitro* in accordance with the invention and then to replace the cells in the organism for example using the technique of Murphy J E *et al* Human Gene Therapy **8**:1867 - 1979, November 1997. Indeed, supplies of infected cells could be maintained to treat a patient over a prolonged period.

Further aspects of the invention and preferred embodiments will be apparent from the appended claims.

The construction of scFv-murine GM-CSF constructs in accordance with the invention and their uses will now be described, by way of example only, with reference to the accompanying drawings Figures 1 to 12 in which:

Fig. 1 is a schematic diagram of constructs in accordance with the invention;

Fig. 2 illustrates the *in vitro* expression of constructs in accordance with the invention;

Fig. 3 is a Western Blot of the results of the expression of various constructs in accordance with the invention;

Fig. 4 illustrates antigen binding by fusion proteins in accordance with the invention;

Fig. 5 illustrates the results of a cell proliferation assay for mGM-CSF bioactivity; and

Fig. 6 illustrates the *in vivo* expression of fusion proteins in accordance with the invention in mice;

Fig.7: shows pAdlox.scFv-IL2 or HuFc(IgG1) generated from pAdlox.scFv-GM-CSF (Whittington *et al* 1998);

Fig. 8 A illustrates the IL2 produced by HeLa cells when infected with recombinant adenovirus of the invention. Fig.8 (B) illustrates the specificity in ELISA against solid phase NIP-BSA and CEA (Calbiochem) of the 48 hour supernatant;

Fig.9 illustrates the specificity in ELISA against solid phase NIP and CEA (Calbiochem) of the 48 hour supernatant;

Fig. 10 illustrates the effects of administration of constructs in accordance with the invention on mice;

Fig. 11 shows the sequence of scFV-mIL2 fusion constructs; and

Fig. 12 shows the sequence of Hu-Fc(IGG1) construct inserted in the above vector.The murine IL2 in the above vector is replaced by the Not1-Xba1 fragment encoding the hinge and Fc region of HU-IgG1 as indicated below.

1. Construction and *in vitro* expression of the scFv.mGM-CSF gene fusion

To determine the optimum cassette for eukaryotic expression of the scFv.mGM-CSF fusion proteins, various vectors were constructed containing different combinations of promoter sequence and leader sequence with scFv.mGM-CSF DNA (Fig. 1). Fig. 1 is a restriction map showing the components of the scFv.mGM-CSF expression cassette. The Hind III/XbaI fragment containing the OM leader sequence, scFv and mGM-CSF DNA was cloned into the multiple cloning site of pAdlox for constructing the recombinant adenoviruses. The CMV promoter is already present within pAdlox (L = th OM or VH1 leader sequence).

2. Antibodies and scFv-mGM-CSF expression cassette

In particular, two scFv antibody fragments are used. One, scFv B1.8, is specific for the haptens 4-hydroxy-3-nitrophenyl acetyl/4-hydroxy-3-nitro-5-iodo-phenylacetyl (NP/NIP) and is derived from a hybridoma (Hawkins RE & Winter G Cell Eur J Immunol.1992; **22**: 867-870). The other, MFE-23, is derived from a phage library and binds to human carcinoembryonic antigen (CEA), which is highly expressed on many tumour types including lung, colorectal and ovarian cancers (Chester KA *et al* Lancet 1994; **343**: 455-456).

To construct a scFv.mGM-CSF expression cassette, the HindIII/Pst I fragment containing the VH1 leader sequence in the eukaryotic expression vector pVAC1 (Stevenson FK *et al* Immunol Rev 1995; 145; 211-228) was replaced with the oncostatin-M leader sequence (OM) (Malik N *et al* Mol Cell Biol 1989; 9: 2847-2853). The engineered signal sequence contains an optimal Kozak start sequence (ACCATGG) (Kozak M Cell 1986; 44: 283-292)

and a Cla I site was introduced at 69 bp to allow for future manipulations. The mGM-CSF DNA was generated by PCR from mGM-CSF cDNA using oligonucleotides to introduce Not I and Xba I restriction sites, to allow cloning into pVAC1, and replace the mGM-CSF signal sequence with a short linker peptide (Ala Ala Ala Gly) to allow fusion to the C-terminus of scFv proteins. The individual scFv DNA's were inserted into the vectors as Pst I/Not I restriction fragments. Vectors to allow comparison of expression using the RSV promoter versus the CMV promoter were generated by excision of the RSV promoter on a Bgl II/Hind III fragment and addition of the CMV promoter from pCEP4 (Invitrogen) as a Acc I/Hind III fragment. An Xba I site present in this fragment was deleted to leave a unique Xba I site within pVAC1. Different constructs were tested to determine optimal expression by transient transfection into 791T or HeLa cells and assay of mGM-CSF in the media after 72 hours using an ELISA kit (Endogen, MA, USA)

The promoters studied were derived from the Rous sarcoma virus (RSV) and cytomegalovirus (CMV). The leader sequences were VH1 from the murine antibody heavy chain (Stevenson FK *et al* Immunol Rev 1995; 145; 211-228) and the OM signal sequence from Oncostatin M (Malik N *et al* Mol Cell Biol 1989; 9: 2847-2853). The expression of the B1.8.mGM-CSF fusion cloned into these vectors was studied in 791T(ATCC:CRL-7798) and HeLa(ATCC:CCL-2) cells. Two cell lines were used to allow for natural differences in the level of expression and protein secretion.

In all experiments, cell lines were cultured in DMEM (Sigma) supplemented with 10% FCS, 100U/ml streptomycin, 100U/ml penicillin and 2mM L-glutamine at 37°C/5% CO_2.

The B1.8.mGM-CSF protein was produced from all the different expression vectors but the level of expression and secretion was higher using the CMV promoter in conjunction with the OM leader sequence in both 791T and HeLa cells (data not shown). The OM leader sequence produced higher levels of B1.8.mGM-CSF secretion than the VH1 leader with both the RSV and CMV promoters(data not shown). Hence the combination of CMV promoter and OM leader sequence was used for subsequent experiments involving the expression of scFv.mGM-CSF proteins in eukaryotic cells. Overall this resulted in 4-fold and 9-fold higher expression than our original vector (pVAC1) in HeLa and 791T cells, respectively.

3. Generation of recombinant adenoviruses

ThescFvs, B1.85 and MFE-23 6, descrobed above, which bind to NIP (4-hydroxy-3-nitro-5-iodo-phenylacetyl) and CEA (carcinoembryonic antigen) respectively were used. The B1.8.mGM-CSF and C23.mGM-CSF gene fusions were subcloned into the Hind III and Xba 1 sites of plasmid pAdlox to yield plasmids pAdlox.B1.8.mGM-CSF and pAdlox.MFE-23.mGM-CSF. The plasmids were individually co-transfected into CRE8 cells with adenovirus Y5 DNA. The Y5 adenovirus, pAdlox plasmid and CRE8 cell line were generously provided by Dr. S Hardy, Somatix (Hardy S *et al* J Virol 1997; 71: 1842-1849). The Y5 adenovirus is replication deficient due to deletions in the viral E1 and E3 genes. The packaging site is directly flanked by loxP sites so that the packaging site is deleted by recombination when the virus is cultured in CRE8 cells, a derivative of 293 cells containing the Cre recombinase gene. Hence there is negative selection for growth of the Y5 virus in CRE8 cells. Growth is restored by recombination with the loxP site in pAdlox, which contains a packaging site (Hardy S *et al* J Virol 1997; 71: 1842-1849). The CRE8 cells promoted recombination between the loxP site in pAdlox.B1.8.mGM-CSF or pAdlox.MFE-23.mGM-CSF and Y5 virus. Cytopathic effects on the CRE8 cells were observed at 10 days post transfection. Control CRE8 cells transfected with either of the pAdlox plasmids or Y5 DNA did not exhibit any cytopathic effects. The recombinant viruses were passaged twice in CRE8 cells to reduce contamination by Y5 virus. Digestion of viral DNA, isolated from cells at each passage, with BsaBI confirmed the presence of the recombinant viruses Y.B1.8.mGM-CSF and Y.MFE-23.mGM-CSF. The titre of virus present after the second passage was approximately 10^{10}/ml cell lysate.

4. Expression of the scFv.mGM-CSF proteins in vitro

The YB1.8.mGM-CSF and YMFE-23.mGM-CSF viruses were used to infect HeLa cells. These were cultured to 70% confluence and, after removal of the culture medium, approximately 10^6 cells were infected in triplicate with viral lysate at a multiplicity of infection (moi) of 1000 in a low volume of cell medium.

After incubation for 1 hour at 37°C/5% CO_2, the viral lysate was removed and the cells washed three times in an excess of culture medium before addition of an appropriate volume of fresh medium (5m./10^6 cells) and further incubation at 37°C/5% Co_2. Culture supernatant was removed at appropriate time points for analysis by ELISA and Western blot.

Media samples were collected at time points up to 48 hours. The samples were assayed for mGM-CSF by ELISA and also examined by Western blot.

To assay for NIP binding MicroTest III Flexible assay plates (Falcon) were coated overnight at 40°C with NIP.BSA conjugate (20mg/ml) in PBS (Hawkins RE & Winter G Cell Eur J Immunol.1992; **22**: 867-870). After blocking for 1 hour with PBS/2%BSA and washing with PBS, culture supernatant diluted in PBS/2%BSA was added to the appropriate wells and incubated for 1 hour at room temperature. After washing three times, bound scFv.mGM-CSF protein was detected using the biotinylated anti-mGM-CSF antibody, Streptavidin-POD conjugate and BM Blue POD substrate according to the protocol in the mGM-CSF ELISA mini-kit (Endogen, MA, USA)

To assay for binding to CEA on the surface of cells, aliquots of LS 174T cells (2 x 10^5), washed with PBS/2% BSA, were incubated with culture supernatants diluted 50:50 in PBS/2% BSA for 1 hour at 40C. Bound scFv.mGM-CSF protein was detected using rabbit anti-mGM-CSF (Sigma, USA) (1/100 in PBS/2%BSA for 30 minutes at 40°C) and FITC conjugated goat anti-rabbit Ig (Sigma, USA)(1/160 in PBS/2%BSA for 30 minutes at 40°C). Between incubations cells were washed three times in PBS/2%BSA. Data was acquired using a Becton Dickinson FACScan and LYSIS II software and analysed using WinMDI 2.4 .

At t=0 there was no detectable scFv.mGM-CSF protein in any of the media samples, confirming that scFv.mGM-CSF present in the viral lysate had been removed by washing after infection of the HeLa cells. B1.8.mGM-CSF and MFE-23.mGM-CSF were detected in the culture supernatants from 4 hours post infection (Figure 2). Specifically, in the work presented in Fig. 2, a Western blot of the samples from the infection confirmed the data obtained by ELISA. The ability of Y.MFE-23.mGM-CSF to direct the expression of the

scFv fusion protein in a variety cells was tested in two murine and five human cell lines. MFE-23.mGM-CSF was produced in all those tested. There was only a minor difference in the level of secretion of MFE-23.mGM-CSF in HeLa compared to HeLa-CEA (HeLa expressing surface CEA following transfection with full length human CEA). The lower efficiency of adenoviral infection of murine cells resulted in lower levels of expression than obtained in human cell lines (data not shown).

A 100ng sample of each scFv.mGM-CSF protein in culture supernatant (as determined by ELISA) was examined by SDS PAGE and Western blot in comparison to 10 ng of recombinant mGM-CSF, expressed in *Escerichia coli* (Sigma, Missouri, USA), to check for any specific proteolytic cleavage in the fusion proteins (Figure 3). Specifically in the work presented in Fig. 3 100 ng MFE-23. mGm-CSF (Lane A), 100ng B1.8.mGm-CSF (Lane B) and 10ng recombinant mGM-CSF, expressed in *E. coli*, (Lane C) were electrophoresed through a 7.5% SDS PAGE gel and transferred to 4.5: nitrocellulose. The proteins were detected using rabbit anti-mGM-CSF according to the methods. The molecular weight markers are Rainbow™ coloured protein markers (Amersham, UK). Protein bands of the correct size (46 kDa) were observed for the scFv.mGM-CSF fusion proteins. There were no other bands representing possible specific proteolytic cleavage products of the fusion protein. As the band of recombinant mGM-CSF is clearly visible, it suggests that any proteolytic products would represent a small percentage (<<10%) of the protein sample.

5. Bioactivity of the scFv.mGM-CSF proteins

Antigen binding activity of B1.8 and MFE-23 scFvs as fusion proteins with mGM-CSF proteins was tested by ELISA and FACS analysis, respectively. Samples of culture supernatant from HeLa cells infected with Y.B1.8.mGM-CSF or Y.MFE-23.mGM-CSF were taken immediately post infection and again after 24 hours incubation. Samples were tested by ELISA against NIP antigen (Figure 4A).

Specifically, in the experiments reflected in Fig. 4A, wells of a 96 well microtitre assay plate were coated with 20µg/ml NIP.BSA conjugate. Uncoated wells were used as a

negative control. Wells were incubated with culture supernatants containing either MFE-23.mGm-CSF or B1.8.mGM-GSF. Bound protein was detected using the biotinylated anti-mGM-CSF antibody from the MGM-CSF ELISA kit (Endogen, MA, USA) as described in the Methods. All samples were assayed in triplicate.

The B1.8.mGM-CSF protein binds to the NIP antigen while, as expected, the MFE-23.mGM-CSF protein does not. The B1.8.mGM-CSF and MFE-23.mGM-CSF proteins, in culture supernatants taken 24 hours post infection, were tested by FACS analysis against LS 174T cells. LS 174T, a human colon carcinoma cell line(ATCC:CL-188), expresses high levels of CEA.

Specifically, 2 x 10^5 LS 175T cells, a colon carcinoma line expressing high levels of CEA, incubated with culture supernatants of either MFE-23.mGM-CSF or B1.8.mGM-CSF. Bound fusion protein was detected with rabbit anti-mGM-CSF (Sigma, UK) and FITC-conjugated goat anti-rabbit Ig (Sigma, UK). The data was acquired using a Becton Dickinson FACScan and LYSIS II software and analysed using WinMDI 2.4.

The FACS analysis (illustrated in Fig. 4B) shows that MFE-23.mGM-CSF protein binds to LS 174T cells while B1.8.mGM-CSF does not.

The ability of mGM-CSF within the scFv.mGM-CSF proteins to promote cell proliferation was demonstrated by a bioassay using FDC-P1 cells. FDC-P1 (ATCC:CRL-12103) is a murine myeloid cell line dependent on IL-3 or mGM-CSF for growth. The B1.8.mGM-CSF and MFE-23.mGM-CSF proteins were compared to recombinant mGM-CSF (Sigma, Missouri, USA), expressed in *E. coli*. HeLa cell culture medium was used as a negative control. Samples between 0.001ng and 50ng were incubated with FDC-P1 cells for 48 hours and proliferation measured by ^{3}H-thymidine incorporation as shown in Fig. 5.

In the experiment the results of which are shown in Fig. 5, aliquots of 5000 FDC-P1 cells, a mGM-CSF-dependent cell line, were incubated with between 50ng to 0.001ng of each of the following: MFE-23.mGM-CSF, B1.8.mGM-CSF and mGM-CSF, which was prepared commercially from protein expressed bacteria, was the positive control. Culture medium was taken from HeLa cells was the negative control. All samples were set up in triplicate. After 48 hours incubation, tritiated thymidine (1:Ci/sample) was added for a further 8

hours incubation. Proliferation was measured using a scintillation counter and is expressed as the number of counts/20 seconds of incorporated thymidine. There was no incorporation by the negative control. The EC_{50} is the concentration of mGM-CSF that elicits a 50% increase in cell growth and hence thymidine incorporation.

The B1.8.mGM-CSF and MFE-23.mGM-CSF proteins appear to have similar bioactivity to recombinant mGM-CSF, as determined by FDC-P1 proliferation. The EC_{50} of the scFv.mGM-CSF proteins, which is the effective concentration of growth factor that elicits a 50% increase in cell growth in a cell based bioassay, is approximately 0.5 ng/ml. Hence both the scFv and mGM-CSF domains of the fusion proteins are bioactive *in vitro*.

6. Expression of MFE-23.mGM-CSF in vivo

To assess the expression of MFE-23.mGM-CSF protein *in vivo*, C57Bl/6 mice were injected intravenously with RMFE-23.mGM-CSF adenovirus purified by CsCl. Mice were injected with between 100ml and 1ml of adenovirus at a titre of approximately 10^{12} pfu/ml in a total volume of 100µl. Sera samples, prior and post infection, were tested by ELISA using the ELISA kit (Endogen for mGM-CSF) (Figure 6). Mice receiving 100ml of virus produced high levels of MFE-23.mGM-CSF (> 1000 ng/ml serum) and were sacrificed after 4 days due to systemic toxicity caused by GM-CSF. The remaining mice injected with 1-10ml of virus produced lower levels of MFE-23.mGM-CSF (~ 2-25 ng/ml serum). Protein expression was detected four days post infection; with levels of expression falling to 0-3 ng/ml serum at 13 days post infection. No MFE-23.mGM-CSF could be detected by 26 days post infection. Serum samples from Mouse 3 were tested for anti-adenoviral antibodies by ELISA using Y.MFE-23.mGM-CSF coated assay plates. 16 Anti-adenoviral antibodies were not present prior to adenoviral infection but could be detected in sera samples from 4 days post-infection (data not shown). Mice No. 1 and 2 were culled at 4 days due to symptoms of systemic toxicity caused by high levels of mGM-CSF. No mGM-CSF could be detected in the remaining mice at 26 days.

The DNA encoding the OM leader sequence and scFv.mGM-CSF proteins, MFE-23.mGM-CSF and B1.8.mGM-CSF, in the eukaryotic expression vector pVAC1 were excised by digestion with HindIII and XbaI. The fragments were subcloned. The recombinant viruses were then constructed by co-transfection of SfiI digested pAdlox.MFE-23.mGM-CSF or pAdlox.B1.8.mGM-CSF with Y5 DNA into CRE8 cells, according to the method described, to yield adenoviruses YMFE-23.mGM-CSF and YB1.8.mGM-CSF (Hardy S *et al* J Virol 1997; 71: 1842-1849). The recombinant viruses were passaged twice in CRE8 cells to reduce contamination with Y5 adenovirus. CRE8 cell lysates were prepared by three rounds of freeze-thawing. The viral titre was approximately 10^9-10^{10}/ml cell lysate. Recombinant adenoviral particles were purified on CsCl gradients and titred by comparison to a control virus in cytopathic effect assays, using standard methods.

7. SDS PAGE and Western Blots

To assess the size of the expressed proteins, and to detect possible degradation, Western blots were used (Towbin, H. *et al*, Proc. Natl. Acad. Sci. 1979, **76**: 4350-4354). Samples were electrophoresed through a 7.5% SDS polyacylamide gel under denaturing conditions using a Mini-Protean II cell (BIORAD, USA). Proteins were transferred to nitrocellulose using the Semi-Phor (Hoefer, USA) semi-dry transfer system. Recombinant mGM-CSF and scFv.mGM-CSF proteins were detected using rabbit anti-mGM-CSF (Sigma, USA)(1/1000 in PBS/0.1% Tween-100), HRP conjugated goat anti-rabbit Ig (Sigma, USA)(1/2000 in PBS/0.1% Tween-100) and then detected using a chemiluminescent detection system according to the protocol supplied with the ECL Western blotting kit (Amersham, UK).

Cell Proliferation Assay

mGM-CSF bioactivity was measured using the mGM-CSF/IL-3 dependent haemopoetic precusor line FDC-P1 (Dexter T. M *et al* J. Exp. Med (1980 **152**: 1036-47). FDC-P1 cells were cultured in DMEM/10%FCS/10% WEHI-3 CM at 37°C in 5% CO_2. The cells were harvested, washed three times in PBS, counted and resuspended at a concentration of 10^5 cells/ml in DMEM/10% FCS. Recombinant mGM-CSF and culture supernatant containing

MFE-23.mGM-CSF or B1.8.mGM-CSF were serially diluted in a final volume of 50ml DMEM/10% FCS in 96-well flat bottomed tissue culture microwell plates. A 50ml aliquot (5000 cells) of FDC-P1 cells was then added to each well. All wells were set up in triplicate. Wells containing no growth factor (HeLa cell culture supernatant) were used as a negative control. Plates were incubated for 48 hours at 37°C in 5%CO_2. Proliferation was measured by incorporation of tritiated thymidine ^{3}H-TdR (1mCi/well). After 8 hours incubation, cells were harvested onto absorbent glass fibre paper and the radioactivity determined using a scintillation counter.

9. Mouse studies

C57Bl/6 mice, bred under specific pathogen-free conditions and maintained according to Home Office approved institutional protocols, were injected in the tail vein with different amounts (total volume 100µl) of pure ΨC23.mGM-CSF diluted in 10mM Tris pH7.5,1mM MgCl2,135mM NaCl,10% glycerol. Blood samples were collected at different time points and the sera assayed for the presence of mGM-CSF using the mouse GM-CSF ELISA kit (Endogen, MA, USA).

The following results provides further examples of scFv-fusions that may be delivered for *in vivo* expression using appropriate viral vectors. The fusions are scFv-IL2 and scFv-HuFc(IgG1). The scFv's used are derived from B1-8 a hybridoma cell line which expresses an antibody with anti-NIP activity and MFE-23 a scFv made from a phage display library (Whittington *et al*, 1998).

10. Preparation of ScFv-IL2 fusion

In order to undertake model mouse experiments the scFv was linked to murine IL2. The IL2 gene was subcloned by PCR from a vector (pVAC4) containing the mouse IL2 gene which was previously cloned from the spleen of a C57BL/6 mouse. The PCR primers incorporate Not1 and Xbal sites in the 5′ and 3′ primers respectively. The PCR fragment encoding IL.2 was cloned into pAdlox.scFv.GM-CSF as a Not1-Xba fragment to produce pAdlox.scFv-IL2. The clones were sequenced and a clone with the correct sequence

selected from further use. Both B1-8-IL.2 and MFE-23-IL2 fusions were made. For details of sequence see Fig 11.

A recombinant adenovirus was then made as previously described by cotransfecting Cre8 cells with pAdlox.scFv-IL2 and Ψ5 viral DNA. After loxP driven recombination and amplification by 3 rounds of lysis and re-infection of Cre8 cells a large viral stock was produced and purified by density gradient purification. The virus was then characterised and the expressed protein assessed after infection of HeLa cells at an MOI of 1. This revealed high level expression of IL2 fusion with retention of the specificity of the scFv portion (Figure 8A & B). The data illustrate high levels of expression of IL2 (comparable to GM-CSF (Whittington *et al* 1998)) and retained specificity.

Figure 8 (A) illustrates the levels of IL2 produced by HeLa cells when infected with recombinant adenovirus at an MOI of 1. The IL2 was measured using an IL2 specific ELISA (Endogen). Fig.8 (B) illustrates the specificity in ELISA against solid phase NIP-BSA and CEA (Calbiochem) of the 48 hour supernatant. The assay uses solid phase antigen with the scFv-IL2 fusion being detected with biotinylated anti-mIL2 antibody. Streptavidin-POD conjugate and BM Blud POD substrate according to the protocol in the mIL2 ELISA mini-kit (Endogen, Cambridge, MA, USA).

11. ScFv-HuFc(IgG1)

This construct was prepared for both B1-8 and MFE-23 using methods similar to those described above. The HuFv(IgG1) was PCR cloned using specific primers with Not1 and Xba11 ends from cDNA derived from human peripheral blood lymphocytes. After cloning into pAdlox.scFv-HuFc(IgG1) clones were DNA sequenced and a clone with the correct sequence identified (Fig.12). Recombinant adenoviruses were then prepared for both scFv's as described above. They were again characterised and used to infect HeLa cells at an MOI of 1. the supernatant was collected at various timepoints and assayed for binding to antigen and presence of the Fc using plates coated with antigen and an anti-human Fc-HRP conjugate (Sigma) (Figure 9). These results indicate that significant amounts of both recombinant antibodies are clearly produced and retain the appropriate specificity.

Figure 9 illustrates the specificity in ELISA against solid phase NIP and CEA (Calbiochem) of the 48 hour supernatant. The assay uses solid phase antigen with the scFv-Fc fusion being detected with anti-human Fc-HRP (Sigma) and BM Blue POD substrate.

Purified Ad5.B1-8-Fc was also used to inject Balb/c mice. The serum was taken at various timepoints and assayed for anti-NIP activity and anti-Hu-Fc activity in an ELISA using NIP coated plates and detecting with an anti-murine-Fc-HRP and BM Blue POD substrate (Figure 10).

In the experiment depicted in Fig. 10, 10^9 Pfu Ad5-B1-8-Fc were injected into the tail vein of three Balb/c mice at day 0 and serum samples taken from the tail veins at days 0, 3, 7, 14, 21, 28. The samples were assays for anti-NIP activity and to assess the immune response to the (human)Fc region by ELISA against NIP-BSA and Human IgG1 (Sigma) - results represent mean of three mice; assays at 1:100 serum dilution for anti-NIP assay and 1:1000 dilution for anti-Fc assay.

As Fig 10 illustrates, the anti-NIP activity initially rises significantly from day 3 to day 7. This is likely to be as a result of accumulation of the scFv-Fc in the serum due to its large size (100kDa). After that the mouse response to the human Fc region is clear and is (presumably responsible for the rapid decline in serum levels (more rapid than with scFv-GM-CSF (Whittington *et al.* 1998) - which is smaller (45kDa)). This would not be apparent if the Hu-Fc construct was used in a person where the scFv-Fc would be anticipated to increase further for a significantly longer time.

Claims

1. A method of producing a fusion protein comprising an antibody portion and a further biologically active portion, the method comprising forming a virus-based construct including a DNA sequence encoding the fusion protein and infecting cells or an organism with the thus constructed virus-based construct whereby the fusion protein is expressed *in vitro* in the cells, or *in vivo* in the organism.

2. A method according to claim 1 in which the virus based construct is based on an adenovirus, retrovirus, adeno-associated virus, herpes simplex virus, or a synthetic virus based particle.

3. A method according to claim 2 in which the adenovirus lacks at least some native sequence.

4. A method according to claim 3 in which the adenovirus lacks all adenovirus sequences.

5. A method according to claim 4 in which the cells or organism are supplied with a helper virus.

6. A method according to claim 2, 3 or 4 in which the adenovirus is ψ5.

7. A method according to claim 2, and in which the virus based construct is based on an retrovirus, in which the retrovirus is a lentivirus.

8. A method according to any preceding claim, and in which the the fusion protein is expressed *in vivo*, in which the organism expresses therapeutic levels of the fusion protein.

9. A method according to claim 5 in which the therapeutic levels of the fusion protein are from about 1 ng ml^{-1} to about 10μg ml^{-1} serum.

10. A method according to claim 9 in which the therapeutic levels are about 1 to 100 ng per ml.

11. A method according to claim 8, 9 or 10 in which the levels of the fusion protein produced are sufficient to produce a therapeutic effect.

12. A method according to claim 11 in which the therapeutic effect is selected from reduction of tumour size, reduction of active agent, reduction of autoimmune disease, reduction of graft v host disease, systemic immune response.

13. A method according to any preceding claim in which the antibody portion comprises a single chain Fv portion.

14. A method according to any preceding claim in which the antibody portion of the protein is selected from or derived from fragments of IgG1, IgG2, IgG3, IgG4 or IgGE.

15. A method according to any preceding claim in which the further biologically active portion is selected from or derived from cytokines, interferons, enzymes, toxins, cell surface ligands for cellular receptors and F_v derived portions of antibodies.

16. A method according to any claim 15 in which the cytokine is selected from GM-CSF, IL-2, IL-4, IL-6, IL-7, IL-12, IL-15.

17. A method according to claim 15 in which the interferon is interferon α or interferon γ.

18. A method according to claim 15 in which the enzyme is carboxypeptidase or β-glucorinidase.

19. A method according to claim 15 in which the toxin is Pseudomonas exotoxin, endotoxin, or ricin.

20. A method according to claim 15 and in which the further biological portion is an enzyme, in which a pro-drug which is converted to an active form by the action of the enzyme.

21. An *in vivo* method according to any preceding claim in which the organism is a mammal.

22. A method according to claim 21 in which the mammal is selected from cattle,, equids, rodents, and humans.

23. A fusion protein comprising a functional antibody portion and a functional further biologically active portion.

24. A fusion protein according to claim 23 in which the further functional biologically active portion comprises at least a portion of GM-CSF or IL-2.

25. A fusion protein consisting of scFv-IL2 or scFv-HuFc(IgG1).

26. An virus-based construct comprising a DNA sequence arranged to express a fusion protein according to claim 23, 24, or 25.

27. An adenovirus based construct according to claim 26 in which the virus-based portion is derived from a Ψ5 adenovirus.

28. A method of expressing a fusion protein according to claim 23, 24 or 25 *in vitro*, the method comprising contacting cells with an virus-based construct including a DNA sequence encoding the fusion protein and infecting cells with the thus-constructed virus-based construct whereby the fusion protein is expressed in the cells.

29. A method according to claim 28 in which the cells are removed from an organism prior to infection with the virus based construct and are later replaced in the organism whereby the fusion protein is expressed in the organism.

30. A method of providing therapeutically useful levels of an fusion protein in an organism, the method comprising infecting the organism with an virus-based construct according to claim 26 or 27.

31. A method according to claim 29 or 30 in which the organism is a mammal.

32. A method according to claim 31 in which the mammal is selected from cattle, equids, rodents and humans.

33. A method or construct or fusion protein according to any preceding claim in which the antibody portion is obtained from a phage display library.

34. Cells infected with a virus based construct or containing a fusion protein construct according to any preceding claim.

FIGURE 1

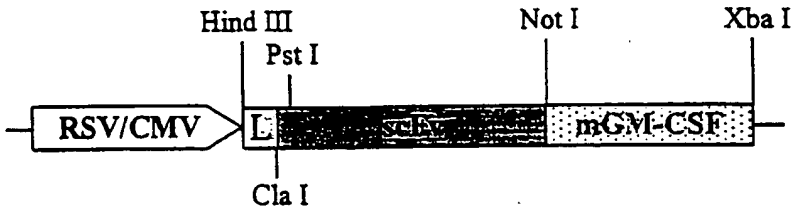


FIGURE 2

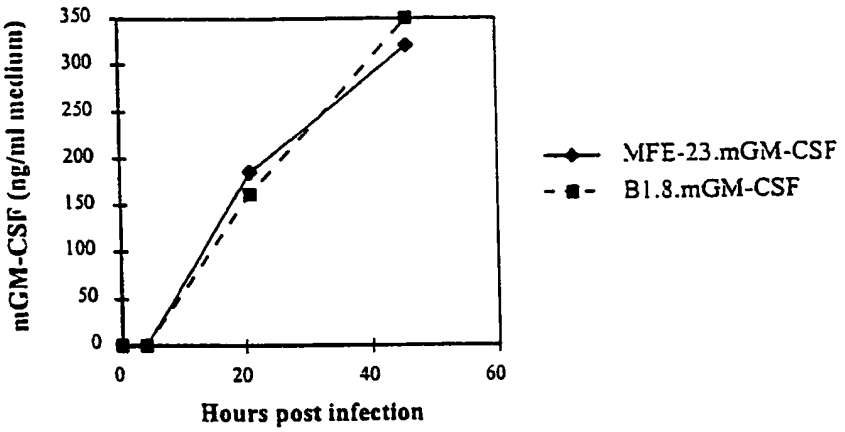


FIGURE 3

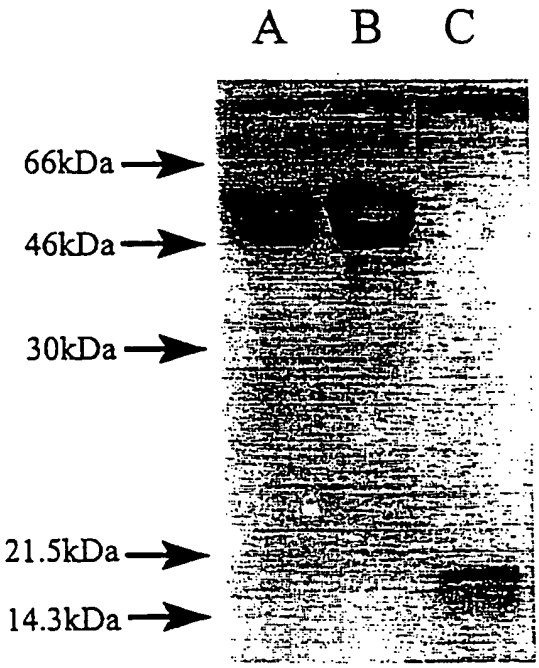


FIGURE 4A

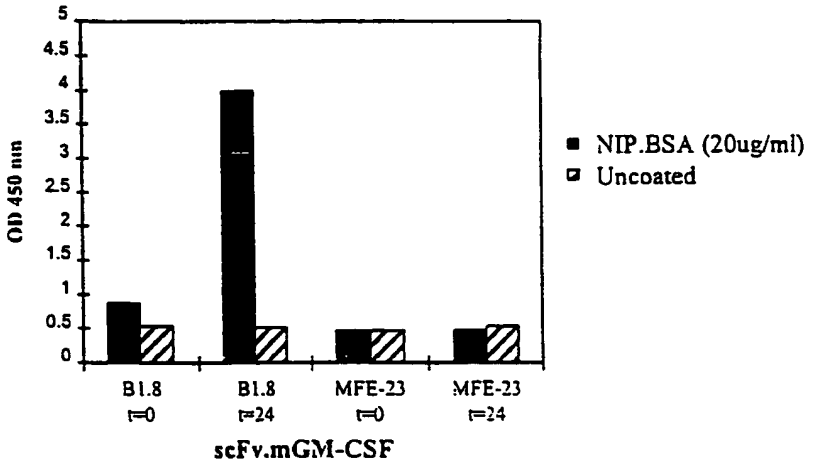


FIGURE 4B

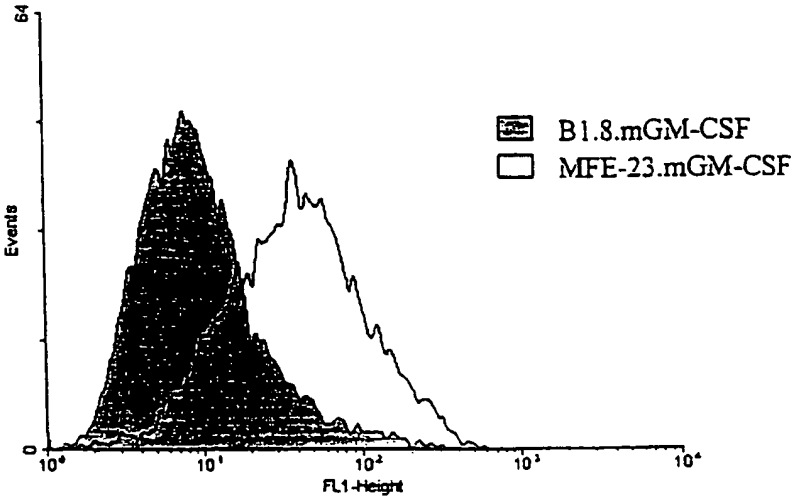


FIGURE 5

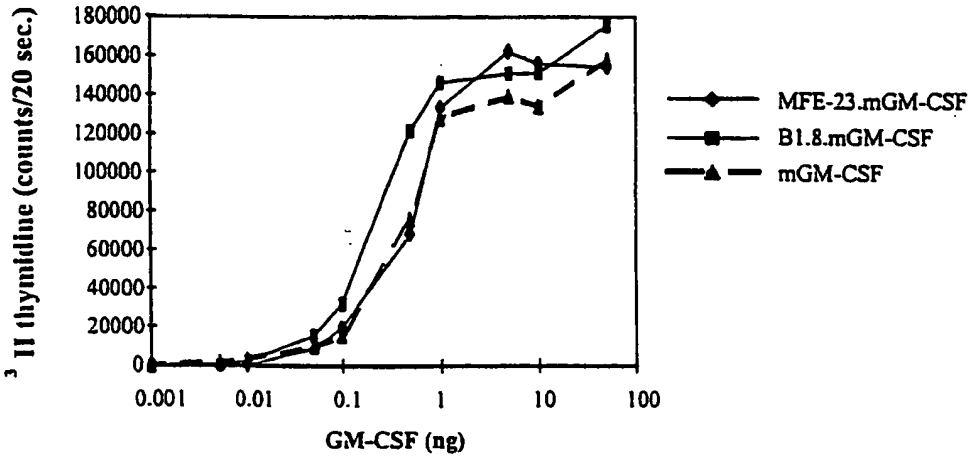


FIGURE 6

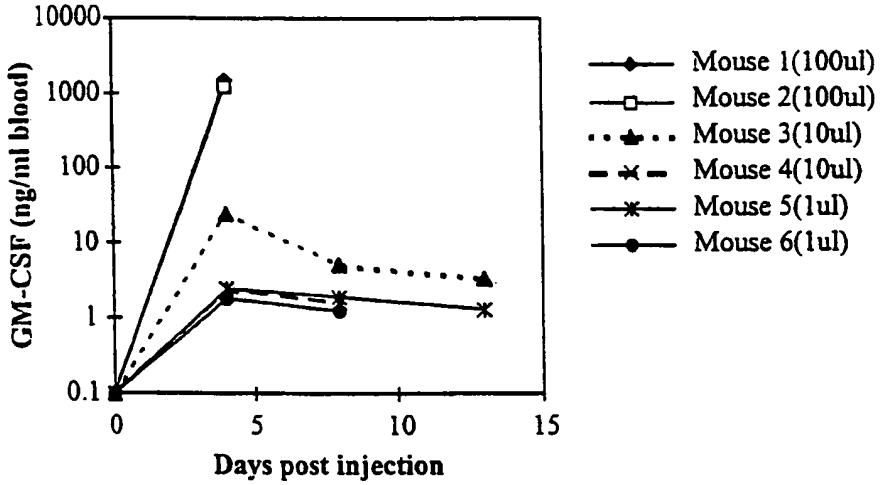


FIGURE 7

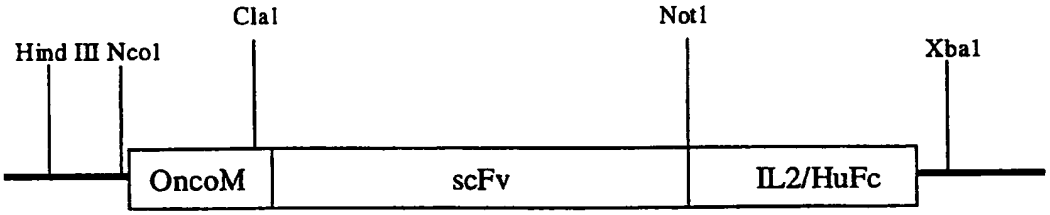


FIGURE 8A

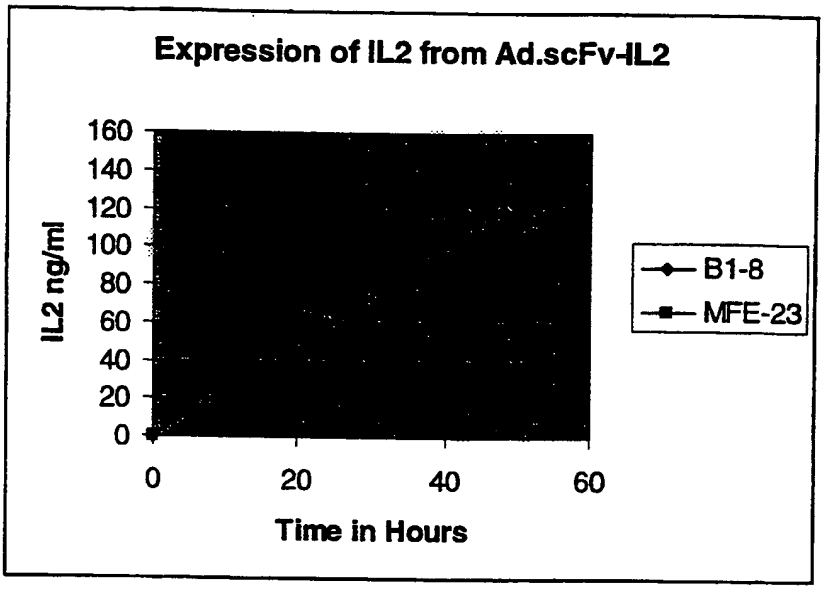


FIGURE 8B

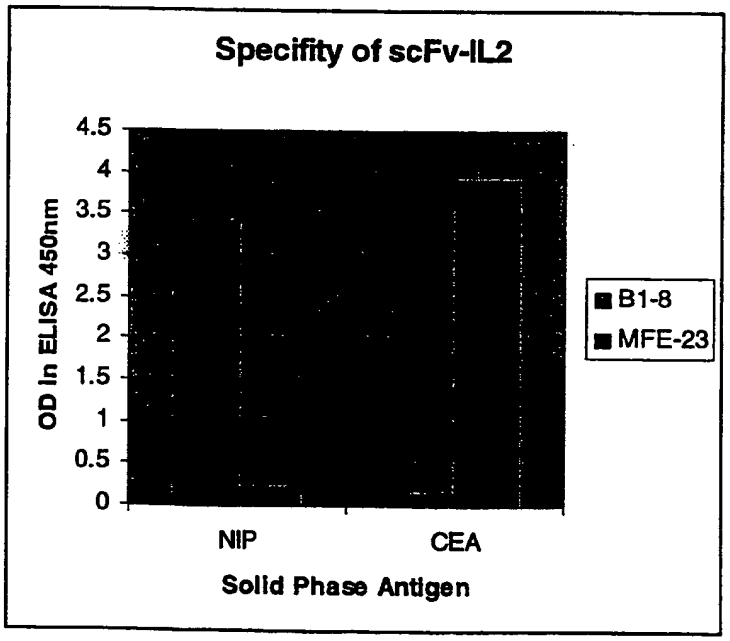


FIGURE 9

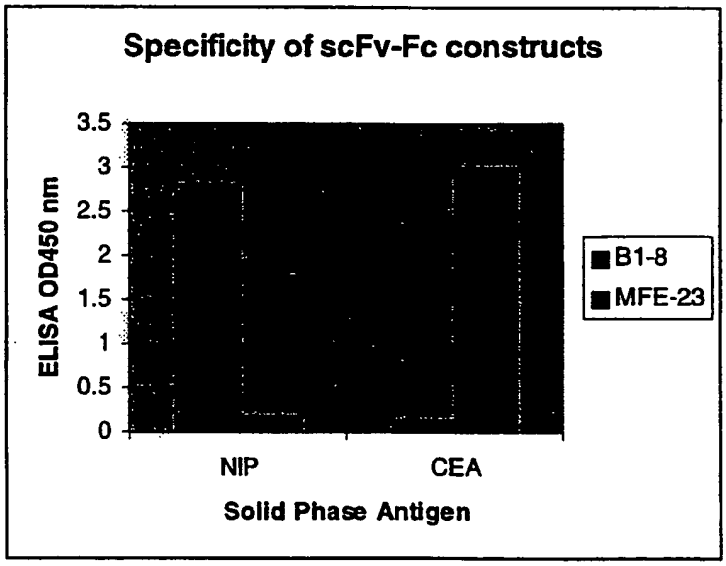

Specificity of scFv-Fc constructs
ELISA OD450 nm
3.5
3
2.5
2
1.5
1
0.5
0
NIP
CEA
Solid Phase Antigen
B1-8
MFE-23

FIGURE 10

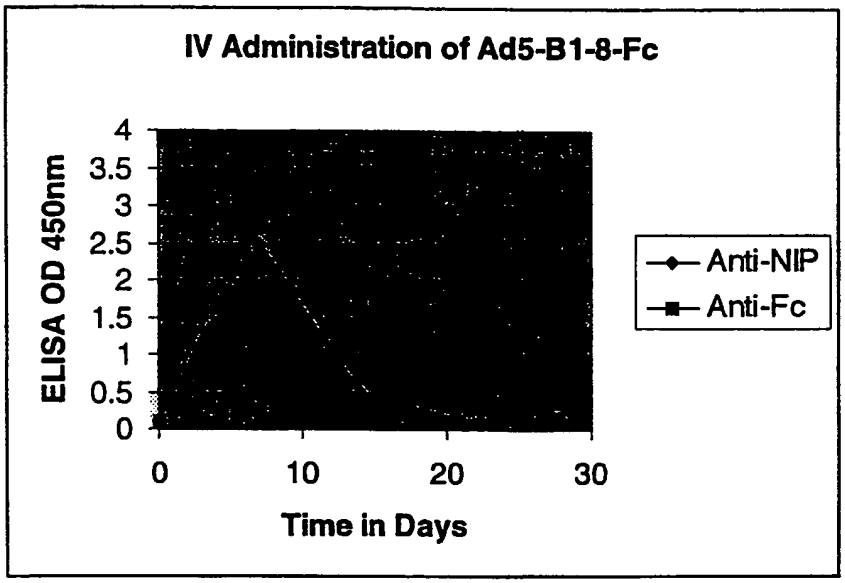
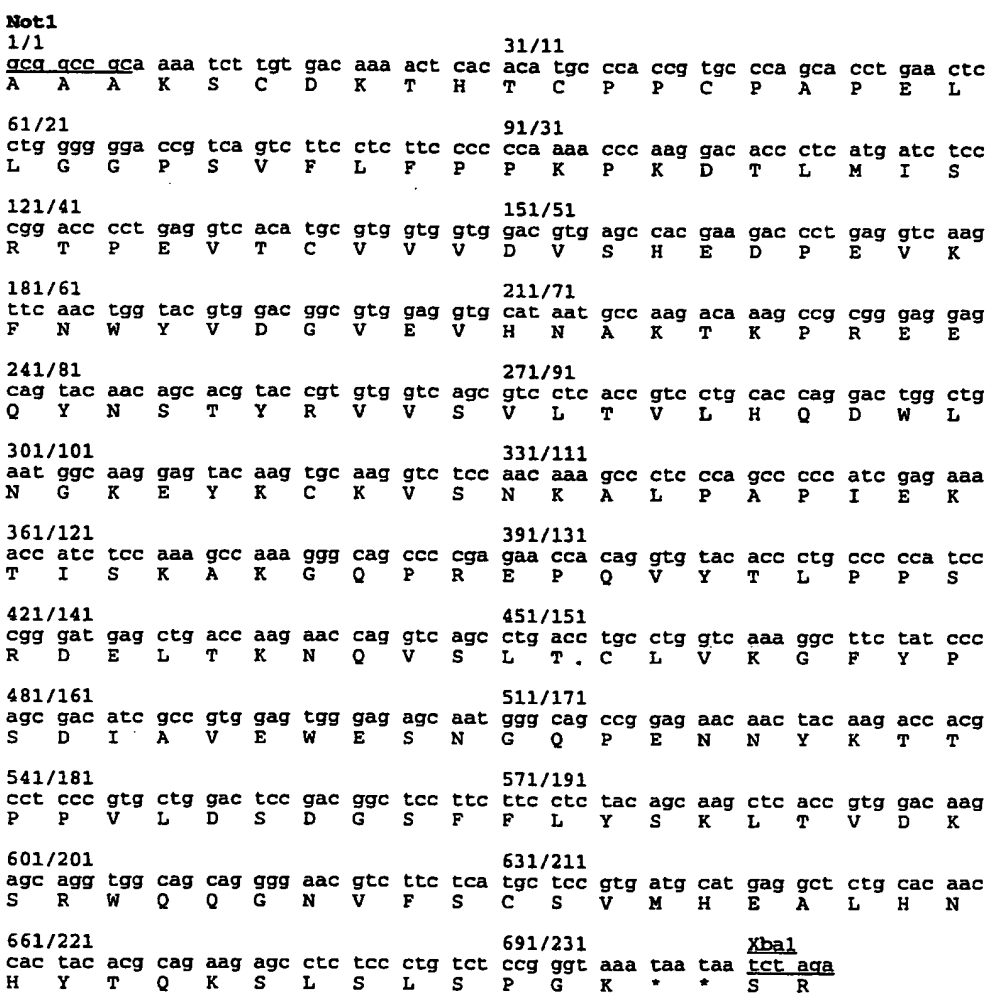

IV Administration of Ad5-B1-8-Fc
ELISA OD 450nm
4
3.5
3
2.5
2
1.5
1
0.5
0
0
10
20
30
Time in Days
Anti-NIP
Anti-Fc

FIGURE 11

```
606/1          Onco M Leader          636/11
aag ctt agc atg gac tgg acc tgg agg gtc ttc tgc ttg ctg gct gtg gcc ccg ggg gcc
K   L   S   M   D   W   T   W   R   V   F   C   L   L   A   V   A   P   G   A

666/21  scFv start                    696/31                          IL2 starts
cac tcc cag gtg cag ctg cag gtc gac ctc gag atc aaa cgg GCG GCC GCA GGT GCA CCC
H   S   Q   V   Q   L   Q   V   D   L   E   I   K   R   A   A   A   G   A   P
                                                    NotI
726/41                                 756/51
ACT TCA AGC TCC ACT TCA AGC TCT ACA GCG GAA GCA CAG CAG CAG CAG CAG CAG CAG CAG
T   S   S   S   T   S   S   S   T   A   E   A   Q   Q   Q   Q   Q   Q   Q   Q

786/61                                 816/71
CAG CAG CAG CAG CAC CTG GAG CAG CTG TTG ATG GAC CTA CAG GAG CTC CTG AGC AGG ATG
Q   Q   Q   Q   H   L   E   Q   L   L   M   D   L   Q   E   L   L   S   R   M

846/81                                 876/91
GAG AAT TAC AGG AAC CTG AAA CTC CCC AGG ATG CTC ACC TTC AAA TTT TAC TTG CCC AAG
E   N   Y   R   N   L   K   L   P   R   M   L   T   F   K   F   Y   L   P   K

906/101                                936/111
CAG GCC ACA GAA TTG AAA GAT CTT CAG TGC CTA GAA GAT GAA CTT GGA CCT CTG CGG CAT
Q   A   T   E   L   K   D   L   Q   C   L   E   D   E   L   G   P   L   R   H

966/121                                996/131
GTT CTG GAT TTG ACT CAA AGC AAA AGC TTT CAA TTG GAA GAT GCT GAG AAT TTC ATC AGC
V   L   D   L   T   Q   S   K   S   F   Q   L   E   D   A   E   N   F   I   S

1026/141                               1056/151
AAT ATC AGA GTA ACT GTT GTA AAA CTA AAG GGC TCT GAC AAC ACA TTT GAG TGC CAA TTC
N   I   R   V   T   V   V   K   L   K   G   S   D   N   T   F   E   C   Q   F

1086/161                               1116/171
GAT GAT GAG TCA GCA ACT GTG GTG GAC TTT CTG AGG AGA TGG ATA GCC TTC TGT CAA AGC
D   D   E   S   A   T   V   V   D   F   L   R   R   W   I   A   F   C   Q   S

1146/181          IL2 ends             1176/191
ATC ATC TCA ACA AGC CCT CAA TAA TCT AGA GCT CGC TGA TCA GCC TCG ACT GTG C
I   I   S   T   S   P   Q   *   S   R   A   R   *   S   A   S   T   V
                                XbaI
```

FIGURE 12


Not1
1/1
gcg gcc gca aaa tct tgt gac aaa act cac
A A A K S C D K T H
31/11
aca tgc cca ccg tgc cca gca cct gaa ctc
T C P P C P A P E L
61/21
ctg ggg gga ccg tca gtc ttc ctc ttc ccc
L G G P S V F L F P
91/31
cca aaa ccc aag gac acc ctc atg atc tcc
P K P K D T L M I S
121/41
cgg acc cct gag gtc aca tgc gtg gtg gtg
R T P E V T C V V V
151/51
gac gtg agc cac gaa gac cct gag gtc aag
D V S H E D P E V K
181/61
ttc aac tgg tac gtg gac ggc gtg gag gtg
F N W Y V D G V E V
211/71
cat aat gcc aag aca aag ccg cgg gag gag
H N A K T K P R E E
241/81
cag tac aac agc acg tac cgt gtg gtc agc
Q Y N S T Y R V V S
271/91
gtc ctc acc gtc ctg cac cag gac tgg ctg
V L T V L H Q D W L
301/101
aat ggc aag gag tac aag tgc aag gtc tcc
N G K E Y K C K V S
331/111
aac aaa gcc ctc cca gcc ccc atc gag aaa
N K A L P A P I E K
361/121
acc atc tcc aaa gcc aaa ggg cag ccc cga
T I S K A K G Q P R
391/131
gaa cca cag gtg tac acc ctg ccc cca tcc
E P Q V Y T L P P S
421/141
cgg gat gag ctg acc aag aac cag gtc agc
R D E L T K N Q V S
451/151
ctg acc tgc ctg gtc aaa ggc ttc tat ccc
L T . C L V K G F Y P
481/161
agc gac atc gcc gtg gag tgg gag agc aat
S D I A V E W E S N
511/171
ggg cag ccg gag aac aac tac aag acc acg
G Q P E N N Y K T T
541/181
cct ccc gtg ctg gac tcc gac ggc tcc ttc
P P V L D S D G S F
571/191
ttc ctc tac agc aag ctc acc gtg gac aag
F L Y S K L T V D K
601/201
agc agg tgg cag cag ggg aac gtc ttc tca
S R W Q Q G N V F S
631/211
tgc tcc gtg atg cat gag gct ctg cac aac
C S V M H E A L H N
661/221
cac tac acg cag aag agc ctc tcc ctg tct
H Y T Q K S L S L S
691/231
ccg ggt aaa taa taa
P G K * *
XbaI
tct aga
S R

PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 :
C12N 15/13, A61K 48/00, C07K 19/00 //
C07K 14/00, 14/535, 14/55, 16/00, 16/30

A3

(11) International Publication Number: **WO 99/36440**

(43) International Publication Date: 22 July 1999 (22.07.99)

(21) International Application Number: PCT/GB99/00152

(22) International Filing Date: 18 January 1999 (18.01.99)

(30) Priority Data:
9800927.7 16 January 1998 (16.01.98) GB

(71) Applicant *(for all designated States except US)*: UNIVERSITY OF BRISTOL [GB/GB]; Senate House, Tyndall Avenue, Bristol BS8 1TH (GB).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: HAWKINS, Robert, Edward [GB/GB]; Christie CRC Research Centre, Wilmslow Road, Manchester M20 4BX (GB). WHITTINGTON, Hayley, Ann [GB/GB]; University Dept. of Oncology, Bristol Oncology Centre, Horfield Road, Bristol BS2 8ED (GB).

(74) Agents: DEAN, John, Paul et al.; Withers & Rogers, Goldings House, 2 Hays Lane, London SE1 2HW (GB).

(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, HR, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published
With international search report.

(88) Date of publication of the international search report:
16 September 1999 (16.09.99)

(54) Title: EXPRESSION OF FUSION PROTEINS

(57) Abstract

This invention provides a method of producing a fusion protein comprising an antibody portion and a further biologically active portion, the method comprising forming a virus–based construct including a DNA sequence encoding the fusion protein and infecting cells or an organism with the thus constructed virus–based construct whereby the fusion protein is expressed in *in vitro* in the cells, or *in vivo* in the organism.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	**ES**	Spain	**LS**	Lesotho	**SI**	Slovenia
AM	Armenia	**FI**	Finland	**LT**	Lithuania	**SK**	Slovakia
AT	Austria	**FR**	France	**LU**	Luxembourg	**SN**	Senegal
AU	Australia	**GA**	Gabon	**LV**	Latvia	**SZ**	Swaziland
AZ	Azerbaijan	**GB**	United Kingdom	**MC**	Monaco	**TD**	Chad
BA	Bosnia and Herzegovina	**GE**	Georgia	**MD**	Republic of Moldova	**TG**	Togo
BB	Barbados	**GH**	Ghana	**MG**	Madagascar	**TJ**	Tajikistan
BE	Belgium	**GN**	Guinea	**MK**	The former Yugoslav Republic of Macedonia	**TM**	Turkmenistan
BF	Burkina Faso	**GR**	Greece			**TR**	Turkey
BG	Bulgaria	**HU**	Hungary	**ML**	Mali	**TT**	Trinidad and Tobago
BJ	Benin	**IE**	Ireland	**MN**	Mongolia	**UA**	Ukraine
BR	Brazil	**IL**	Israel	**MR**	Mauritania	**UG**	Uganda
BY	Belarus	**IS**	Iceland	**MW**	Malawi	**US**	United States of America
CA	Canada	**IT**	Italy	**MX**	Mexico	**UZ**	Uzbekistan
CF	Central African Republic	**JP**	Japan	**NE**	Niger	**VN**	Viet Nam
CG	Congo	**KE**	Kenya	**NL**	Netherlands	**YU**	Yugoslavia
CH	Switzerland	**KG**	Kyrgyzstan	**NO**	Norway	**ZW**	Zimbabwe
CI	Côte d'Ivoire	**KP**	Democratic People's Republic of Korea	**NZ**	New Zealand		
CM	Cameroon			**PL**	Poland		
CN	China	**KR**	Republic of Korea	**PT**	Portugal		
CU	Cuba	**KZ**	Kazakstan	**RO**	Romania		
CZ	Czech Republic	**LC**	Saint Lucia	**RU**	Russian Federation		
DE	Germany	**LI**	Liechtenstein	**SD**	Sudan		
DK	Denmark	**LK**	Sri Lanka	**SE**	Sweden		
EE	Estonia	**LR**	Liberia	**SG**	Singapore		

INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB 99/00152

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/13 A61K48/00 C07K19/00 //C07K14/00,C07K14/535, C07K14/55,C07K16/00,C07K16/30

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07K A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	VERMA I M ET AL: "Gene therapy -- promises, problems and prospects [news]." NATURE, (1997 SEP 18) 389 (6648) 239-42. JOURNAL CODE: NSC. ISSN: 0028-0836., XP002910793 ENGLAND: United Kingdom see the whole document ---	1-22, 26-34
X	CHESTER K A ET AL: "Clinical issues in antibody design." TRENDS IN BIOTECHNOLOGY, (1995 AUG) 13 (8) 294-300. REF: 54 JOURNAL CODE: ALJ. ISSN: 0167-7799., XP002106262 ENGLAND: United Kingdom cited in the application see page 297, left-hand column see page 298 see page 299, right-hand column ---	1,2, 8-22,26, 28-34
	-/--	

[X] Further documents are listed in the continuation of box C. [ ] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
30 June 1999	09. 07. 99

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Mennessier, T

INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB 99/00152

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	KOZARSKY K F ET AL: "Gene therapy: adenovirus vectors." CURRENT OPINION IN GENETICS AND DEVELOPMENT, (1993 JUN) 3 (3) 499-503. REF: 31 JOURNAL CODE: BJC. ISSN: 0959-437X., XP002107739 ENGLAND: United Kingdom see page 499 - page 500 see page 502 ---	1-6, 8-22, 28-34
X	WATKINS, S. J. ET AL: "The 'adenobody' approach to viral targeting: specific and enhanced adenoviral gene delivery" GENE THER. (1997), 4(10), 1004-1012 CODEN: GETHEC;ISSN: 0969-7128, XP002106261 see page 1004 see page 1010 see page 1011, left-hand column ---	23,33
X	HORNICK J L ET AL: "Chimeric CLL-1 antibody fusion proteins containing granulocyte-macrophage colony-stimulating factor or interleukin - 2 with specificity for B-cell malignancies exhibit enhanced effector functions while retaining tumor targeting properties." BLOOD, (1997 JUN 15) 89 (12) 4437-47. JOURNAL CODE: A8G. ISSN: 0006-4971., XP002106263 United States cited in the application see page 4437 ---	23,24
X	CHESTER, KERRY A. ET AL: "Opportunities with phage technology and antibody engineering of fusion proteins" ADV. DRUG DELIVERY REV. (1996), 22(3), 303-313 CODEN: ADDREP;ISSN: 0169-409X, XP002106264 cited in the application see page 306 see page 309, right-hand column - page 310 ---	23,33
X	GILLILAND L K ET AL: "Rapid and reliable cloning of antibody variable regions and generation of recombinant single chain antibody fragments." TISSUE ANTIGENS, (1996 JAN) 47 (1) 1-20. JOURNAL CODE: VSV. ISSN: 0001-2815., XP002035286 Denmark see page 4, right-hand column - page 5, left-hand column; figure 2 ---	25

-/--

INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB 99/00152

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	CHESTER K A ET AL: "Phage libraries for generation of clinically useful antibodies." LANCET, (1994 FEB 19) 343 (8895) 455-6. JOURNAL CODE: L0S. ISSN: 0140-6736., XP002106266 ENGLAND: United Kingdom see the whole document	33
X	SAVAGE ET AL: "A recombinant single chain antibody interleukin-2 fusion protein" BR. J. CANCER, vol. 67, 1993, pages 304-310, XP002106265 cited in the application see page 304 see page 308 - page 309	23-25
P,X	CONNELLY R J ET AL: "Mitogenic properties of a bispecific single-chain Fv-Ig fusion generated from CD2-specific mAb to distinct epitopes." INTERNATIONAL IMMUNOLOGY, (1998 DEC) 10 (12) 1863-72. JOURNAL CODE: AY5. ISSN: 0953-8178., XP002106267 ENGLAND: United Kingdom see page 1863 - page 1864	25
P,X	HAWKINS R E ET AL: "Antibodies: from genes to targeted cancer gene therapy [editorial]." GENE THERAPY, (1998 DEC) 5 (12) 1581-3. JOURNAL CODE: CCE. ISSN: 0969-7128., XP002106269 ENGLAND: United Kingdom see the whole document	1-34
P,X	WHITTINGTON H A ET AL: "Recombinant adenoviral delivery for in vivo expression of scFv antibody fusion proteins." GENE THERAPY, (1998 JUN) 5 (6) 770-7. JOURNAL CODE: CCE. ISSN: 0969-7128., XP002106268 ENGLAND: United Kingdom see the whole document	1-34

INTERNATIONAL SEARCH REPORT

International application No.
PCT/GB 99/00152

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Although claims 1-22, 30-32 and 33 (partly) are directed to a method of treatment by gene therapy of the human/animal body, the search has been carried out and based on the alleged effects of the virus-based construct.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.